No. 9900018

# THE UNITED STATES OF AMERICA

TO ALL TO WHOM THESE PRESENTS SHALL COME:

## Cebeco Zaden B.V.

Whereas, THERE HAS BEEN PRESENTED TO THE

Secretary of Agriculture

AN APPLICATION REQUESTING A CERTIFICATE OF PROTECTION FOR AN ALLEGED DISTINCT VARIETY OF SEXUALLY REPRODUCED, OR TUBER PROPAGATED, PLANT, THE NAME AND DESCRIPTION OF WHICH ARE CONTAINED IN THE APPLICATION AND EXHIBITS, A COPY OF WHICH IS HEREUNTO ANNEXED AND MADE A PART HEREOF, AND THE VARIOUS REQUIREMENTS OF LAW IN SUCH CASES MADE AND PROVIDED HAVE BEEN COMPLIED WITH, AND THE TITLE THERETO IS, FROM THE RECORDS OF THE PLANT VARIETY PROTECTION OFFICE, IN THE APPLICANT(S) INDICATED IN THE SAID COPY, AND WHEREAS, UPON DUE EXAMINATION MADE, THE SAID APPLICANT(S) IS (ARE) ADJUDGED TO BE ENTITLED TO A CERTIFICATE OF PLANT VARIETY PROTECTION UNDER THE LAW.

NOW, THEREFORE, THIS CERTIFICATE OF PLANT VARIETY PROTECTION IS TO GRANT UNTO THE SAID APPLICANT(S) AND THE SUCCESSORS, HEIRS OR ASSIGNS OF THE SAID APPLICANT(S) FOR THE TERM OF TWENTY YEARS FROM THE DATE OF THIS GRANT, SUBJECT TO THE PAYMENT OF THE REQUIRED FEES AND PERIODIC REPLENISHMENT OF VIABLE BASIC SEED OF THE VARIETY IN A PUBLIC REPOSITORY AS PROVIDED BY LAW, THE RIGHT TO EXCLUDE OTHERS FROM SELLING THE VARIETY, OR OFFERING IT FOR SALE, OR REPRODUCING IT, OR PORTING IT, OR EXPORTING IT, OR CONDITIONING IT FOR PROPAGATION, OR STOCKING IT FOR ANY OF THE VE PURPOSE , OR USING IT IN PRODUCING A HYBRID OR DIFFERENT VARIETY THEREFROM, TO THE EXTENT DED BY THE PLANT VARIETY PROTECTION ACT. IN THE UNITED STATES SEED OF THIS VARIETY (1) E SOLD BY VARIETY NAME ONLY AS A CLASS OF CERTIFIED SEED AND (2) SHALL CONFORM TO THE N F GENERATIONS SPECIFIED BY THE OWNER OF THE RIGHTS. (84 STAT. 1542, AS AMENDED, 7 U.S.C. 2321 ET

*PEA, FIELD*

*'Toledo'*

*In Testimony Whereof, I have hereunto set my hand and caused the seal of the Plant Variety Protection Office to be affixed at the City of Washington, D.C. this eighteenth day of September, in the year of our Lord two thousand.*

PLANT VARIETY PROTECTION OFFICE · U.S. DEPARTMENT OF AGRICULTURE

*Attest:*

*Commissioner*
*Plant Variety Protection Office*
*Agricultural Marketing Service*

*Secretary of Agriculture*

REPRODUCE LOCALLY. *Include form number and date on all reproductions.* FORM APPROVED - OMB NO. 05

U.S. DEPARTMENT OF AGRICULTURE
AGRICULTURAL MARKETING SERVICE
SCIENCE AND TECHNOLOGY DIVISION - PLANT VARIETY PROTECTION OFFICE

# APPLICATION FOR PLANT VARIETY PROTECTION CERTIFICATE

*(Instructions and information collection burden statement on reverse)*

*The following statements are made in accordance with the Privacy 1974 (5 U.S.C. 552a) and the Paperwork Reduction Act (PRA) of 1*

*Application is required in order to determine if a plant variety pro certificate is to be issued (7 U.S.C. 2421). Information is held conf until certificate is issued (7 U.S.C. 2426).*

1. NAME OF APPLICANT(S) *(as it is to appear on the Certificate)*
CEBECO ZADEN B.V.

2. TEMPORARY DESIGNATION OR EXPERIMENTAL NUMBER
CEBECO 1145

3. VARIETY NAME
TOLEDO

4. ADDRESS *(Street and No., or R.F.D. No., City, State, and ZIP Code, and Country)*
P.O.Box 10000
5250 GA Vlijmen - Holland

RWS 10-2-98

5. TELEPHONE *(include area code)*
+31 73 51 88 ~~555~~ 570

6. FAX *(include area code)*
+31 73 51 88 ~~666~~ 572

**FOR OFFICIAL USE ONLY**

PVPO NUMBER
9900018

FILING DATE
10-13-98

7. GENUS AND SPECIES NAME
Pisum sativum L.

8. FAMILY NAME *(Botanical)*
Leguminosae

9. CROP KIND NAME *(Common name)*
Field pea

FEES RECEIVED
FILING AND EXAMINATION FEE:
$ 2450.00
DATE 7/6/98

CERTIFICATION FEE:
$ 300
DATE 9/8/00

10. IF THE APPLICANT NAMED IS NOT A "PERSON", GIVE FORM OF ORGANIZATION *(corporation, partnership, association, etc.)* *(Common name)*

11. IF INCORPORATED, GIVE STATE OF INCORPORATION

12. DATE OF INCORPORATION

13. NAME AND ADDRESS OF APPLICANT REPRESENTATIVE(S), IF ANY, TO SERVE IN THIS APPLICATION AND RECEIVE ALL PAPERS

RWS 10-2-98
~~I.S.I.~~ Ir. Bert Scholte, P.O.Box 10000, 5250 GA Vlijmen - Holland
~~POB 168~~
~~Halsey, Oregon 97348 - USA~~

14. TELEPHONE *(include area code)*
+1 541 369 2251

15. FAX *(include area code)*
+1 541 369 2640

16. CHECK APPROPRIATE BOX FOR EACH ATTACHMENT SUBMITTED *(Follow instructions on reverse)*

a. ☒ Exhibit A. Origin and Breeding History of the Variety
b. ☒ Exhibit B. Statement of Distinctness
c. ☒ Exhibit C. Objective Description of the Variety
d. ☒ Exhibit D. Additional Description of the Variety *(Optional)*
e. ☒ Exhibit E. Statement of the Basis of the Applicant's Ownership
f. ☒ Voucher Sample *(2,500 viable untreated seeds or, for tuber propagated varieties verification that tissue culture will be deposited and maintained in an approved public repository)*
g. ☒ Filing and Examination Fee ($2,450), made payable to "Treasurer of the United States" *(Mail to PVPO)*

17. DOES THE APPLICANT SPECIFY THAT SEED OF THIS VARIETY BE SOLD BY VARIETY NAME ONLY, AS A CLASS OF CERTIFIED SEED? *(See Section 83(a) of the Plant Variety Protection A*
☒ YES *(If "yes," answer items 18 and 19 below)* ☐ NO *(If "no," go to item 20)*

18. DOES THE APPLICANT SPECIFY THAT SEED OF THIS VARIETY BE LIMITED AS TO NUMBER OF GENERATIONS?
☒ YES ☐ NO

19. IF "YES" TO ITEM 18, WHICH CLASSES OF PRODUCTION BEYOND BREEDER SE
☒ FOUNDATION ☒ REGISTERED ☒ CERTIFIED 1 gen. on.

20. HAS THE VARIETY OR A HYBRID PRODUCED FROM THE VARIETY BEEN RELEASED, USED, OFFERED FOR SALE, OR MARKETED IN THE U.S. OR OTHER COUNTRIES?
☐ YES *(If "yes," give names of countries and dates)* ☒ NO per letter of 3/3/1999 MAH 7/17/2000

21. The applicant(s) declare that a viable sample of basic seed of the variety will be furnished with application and will be replenished upon request in accordance with such regulations as may be applicable, or for a tuber propagated variety a tissue culture will be deposited in a public repository and maintained for the duration of the certificate.

The undersigned applicant(s) is(are) the owner(s) of this sexually reproduced or tuber propagated plant variety, and believe(s) that the variety is new, distinct, uniform, and stable as required in Section 42, and is entitled to protection under the provisions of Section 42 of the Plant Variety Protection Act.

Applicant(s) is(are) informed that false representation herein can jeopardize protection and result in penalties.

SIGNATURE OF APPLICANT *(Owner(s))*
CEBECO ZADEN B.V. Vlijmen, Holland

NAME *(Please print or type)*
Ir.B.Scholte

CAPACITY OR TITLE
Head Registration Dept.

DATE
May 11, 1998

SIGNATURE OF APPLICANT *(Owner(s))*

NAME *(Please print or type)*

CAPACITY OR TITLE

DATE

STD-470 (03-96) *(Previous editions are to be destroyed)*

*(See reverse for instructions and information collection burden state*

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CEBECO ZADEN B.V.

EXHIBIT A ( revised)

## Origin and Breeding History of Cebeco 1145 ( Toledo) ( Pisum sativum L.)

### 1. Genealogy

Cebeco 1145 ( Toledo) is derived form the following crossing:

The pedigree of Toledo is Lu-Y * Solara, as stated in the original application file. Lu-Y is a selection from the Czech Republic which never made it to a commercial available variety.

} copied from faxed of 7-4-00 RWS 8-11-00

In 1992 a single plant was selected in the F3, followed by line selection and replicated yield trials.

The breeding process was carried out at our breeding station in Lelystad in the Center of the Netherlands. Our breeding station is situated on 150 ha reclaimed land on heavy clay soil and is equipped with all the necessary facilities including lab facilities and greenhouses.

### 2. Breeding goals

The breeding goals were:

| Generation | Criteria for selection |
|---|---|
| **F4 plant selection** | Straw stiffness<br>Plant length |
| **F5 line selection** | Straw stiffness<br>Maturity<br>Resistance to - mildew<br>- Botrytis |
| **F6 yield trials** | Yield<br>Straw Stiffness<br>Seed size |
| **F7 yield trials and further** | Yield<br>Protein content |

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CEBECO ZADEN B.V.

**Exhibit A-bis, revised**

**Evidence of Uniformity and Stability of Cebeco 1145 ( Toledo) ( Pisum sativum L.)**

Cebeco 1145 ( Toledo) has been tested in official DUS-trials in the the Netherlands in the years 1997-1998. Based on the results from these trials the official authorities have concluded the following:

1. During the testing years no variants were observed, thus confirming the Uniformity of Cebeco 1145 ( Toledo).

2. Over the 2 years Cebeco 1145 ( Toledo) has shown to be a Stable variety.

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CEBECO ZADEN B.V.

**Exhibit B- revised**

## Statement of Distinctness of of CEBECO 1145 ( TOLEDO) ( Pisum sativum L.)

Cebeco 1145 ( Toledo) has been found Distinct from all other known varieties but is most similar to Solara. However Cebeco 1145 ( Toledo) can be distinguished from Solara in the following characteristics:

| Character | | Cebeco 1145 ( Toledo) | Solara |
|---|---|---|---|
| **TGW** | 1993 | 284 | 362 |
| | 1994 | 254 | 302 |
| | 1995 | 277 | 313 |
| | 1996 | 274 | 330 |
| **Standing ability** | | | |
| | 1993 | 2.6 | 4.1 |
| | 1994 | 3.7 | 6.1 |
| | 1995 | 2.6 | 5.1 |
| | 1996 | 3.0 | 5.1 |
| **Protein content** | | | |
| | 1992 | 27.1 | 27.5 |
| | 1993 | 26.0 | 26.2 |
| | 1994 | 26.0 | 26.4 |
| | 1995 | 26.2 | 26.4 |

2. **Exhibit B. Statement of Distinctness.**

- The protein I am referring to in Exhibit B-revised dd. 03-03-99 is storage protein.

  Protein Content of Pea is measured by NIRS at our Laboratory in Lelystad. The equipment used is the Bran & Luebbe InfraLyzer 450 (700-2600 nm), which is a fixed filter machine. For the protein content calibration of Pea, 4 filters are used. The sample used is approx. 10 gr. of Pea meal, grinded on a Retch mill at 0.75 mm. The Control varieties Solara and Baccara are used to correct for year or site interactions. In general the protein content is calculated on the basis of 4 years of result in 3 replications.

- Standing ability. The scale used for Standing ability runs from 1 – 9; 1 being 100 % standing upright and 9 being 100 % lodging.

FORM APPROVED. OMB NO. 40-R371

FORM GR-470-14
(5-15-74)

UNITED STATES DEPARTMENT OF AGRICULTURE
AGRICULTURAL MARKETING SERVICE
GRAIN DIVISION
HYATTSVILLE, MARYLAND 20782

EXHIBIT
(Pea)

# OBJECTIVE DESCRIPTION OF VARIETY

**PEA *(PISUM SATIVUM)***

NAME OF APPLICANT(S)
CEBECO ZADEN BV.

ADDRESS *(Street and No. or R.F.D. No., City, State, and ZIP Code)*
POB 10000
5250 GA Vlijmen - Holland

VARIETY NAME OR TEMPORARY DESIGNATION
CEBECO 1145 (TOLEDO)

FOR OFFICIAL USE ONLY
PVPO NUMBER 9900018

Place the appropriate number that describes the varietal character in the boxes below.
Place a zero in first box (e.g. [0][8][9] or [0][9]) when number is either 99 or less or 9 or less.

**1. TYPE:**

[2] 1 = GARDEN 2 = FIELD 3 = EDIBLE-PODDED

**2. MATURITY:**

[13] Node number of first bloom: [ ][ ][ ] No. of days to processing [ ][ ][ ][ ] Heat Units

[ ][ ] No. of days *Earlier* than . . . [ ] 1 = ALASKA WR 2 = THOMAS LAXTON WR 3 = LITTLE MARVEL

[ ][ ] No. of days *Later* than . . . . [7] 4 = WANDO 5 = ALDERMAN WR 6 = AUSTRIAN WINTER

**3. PLANT HEIGHT:**

[8][7] CM. HIGH

[ ][ ] Cm. *Shorter* than . . . . . [ ] 1 = ALASKA WR 2 = THOMAS LAXTON WR 3 = LITTLE MARVEL

[1][4] Cm. *Taller* than . . . . . [ ] 4 = WANDO 5 = ALDERMAN WR 6 = AUSTRIAN WINTER 7 = Solar

**4. VINE:**

[1] Habit: 1 = DETERMINATE 2 = INDETERMINATE [2] Stockiness: 1 = SLIM (Alaska) 2 = MEDIUM (Thomas Laxton WR) 3 = HEAVY (Alderman)

[3] Branching: 1 = NONE (Alaska) 2 - 1–2 BRANCHES (Little Marvel) 3 = MORE THAN 2 BRANCHES (Dwarf Gray Sugar

[2] Internodes: 1 = STRAIGHT 2 = ZIG ZAG [2][3] NUMBER OF NODES

**5. LEAFLETS:** NOT PRESENT

[ ] Color: 1 = LIGHT GREEN (Alaska WR) 2 = MED. GREEN (Thomas Laxton WR) 3 = DARK GREEN (Alderman) 4 = OTHER (Specify)

[ ] Wax: 1 = NONE 2 = LIGHT 3 = MEDIUM 4 = HEAVY [ ] 1 = NOT MARBLED 2 = MARBLED (Alaska)

[ ] Number of leaflet pairs: 1 = NOT PAIRED 2 = ONE 3 = TWO 4 = THREE OR MORE

**6. STIPULES:**

[2] 1 = LACKING 2 = PRESENT [2] 1 = NOT CLASPING 2 = CLASPING

[2] 1 = NOT MARBLED 2 = MARBLED [X] Size (Compared with leaflets): 1 = SMALLER 2 = SAME 3 = LARGER

[X] Color (Compared with leaflets): 1 = LIGHTER 2 = SAME 3 = DARKER LEAFLETS NOT PRESENT

**7. FLOWER COLOR:**

[2] VENATION [1] STANDARD [1] WING [6] KEEL 1 = WHITE 2 = GREENISH 3 = LAVENDER 4 = PURPLE 5 = RED 6 = OTHER (Specify) creamy

CEBECO 1145 (TOLEDO)

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**8. PODS:**

[2] Shape: 1 = STRAIGHT 2 = SLIGHTLY CURVED 3 = CURVED

[2] End: 1 = POINTED (Alderman) 2 = BLUNT (Alaska)

[2] Color: 1 = LIGHT GREEN (Alaska WR) 2 = MEDIUM GREEN 3 = DARK GREEN (Alderman) 4 = OTHER (Specify) _____

[1] Surface: 1 = SMOOTH 2 = ROUGH

[2] Surface: 1 = SHINY 2 = DULL

[2] Borne: 1 = SINGLE 2 = DOUBLE 3 = SINGLE AND DOUBLE 4 = SINGLE, DOUBLE, & TRIPEE 5 = DOUBLE & TRIPLE 6 = TRIPLE 7 = OTHER (Specify) _____

[6][6] CM. LENGTH

[1][3] MM. WIDTH (Between sutures)

[0][8] NO. SEEDS PER POD

**9. SEEDS (95–100 Tenderometer):**

[4] Color: 1 = LIGHT GREEN 2 = GREEN 3 = DARK GREEN 4 = OTHER (Specify) light-medium

Seive: %

| 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | AVERAGE |
|---|---|---|---|---|---|---|---|---|
| [ ] | [ ] | [ ] | [ ] | [ ] | [ ] | [ ] | [ ] | [ ] |

SEEDS (Dry, Mature):

[4] Shape: 1 = FLATTENED 2 = ANGULAR 3 = OVAL 4 = ROUNDED

[1] Surface: 1 = SMOOTH 2 = DIMPLED 3 = WRINKLED

[2] Surface: 1 = SHINY 2 = DULL

[1] Color Pattern: 1 = MONOCOLOR 2 = MOTTLED 3 = STRIPED 4 = DOTTED

[4] Primary Color:

[ ] Secondary Color:

1 = CREAMY–WHITE 2 = CREAM & GREEN 3 = LIGHT GREEN 4 = MEDIUM GREEN 5 = DARK GREEN 6 = BLUE–GREEN 7 = YELLOW 8 = BROWN 9 = RED 10 = GRAY 11 = BLACK

[1] Hilum Floor Color: 1 = WHITE 2 = TAN 3 = BLACK

[1] Cotyledon Color: 1 = GREEN 2 = YELLOW 3 = ORANG

per letter of 3/3/1999
MAH 7/17/2000

[2][8] GRAMS PER 100 SEEDS

**10. DISEASE: (0 = Not Tested; 1 = Susceptible; 2 = Resistant)**

[2] FUSARIUM WILT race 1

[0] NEAR–WILT

[1] DOWNY MILDEW

[1] ASCOCHYTA BLIGHT race C

[1] POWDERY MILDEW

[0] BACTERIAL BLIGHT

[1] MOSAIC

[0] PEA ENATION MOSAIC

[0] YELLOW BEAN MOSAIC

[ ] OTHER (Specify) _____

**11. INSECT: (0= Not Tested; 1 = Susceptible; 2 = Resistant)**

[ ] APHIDS

[ ] OTHER (Specify) _____

**12. INDICATE WHICH VARIETY MOST CLOSELY RESEMBLES THAT SUBMITTED**

| CHARACTER | NAME OF VARIETY | CHARACTER | NAME OF VARIETY |
|---|---|---|---|
| Leafiness | | Fresh Seed Color | |
| Leaf Color | | Mature Seed Color | |
| Pod Color | | Seed Shape | |
| Pod Shape | | Plant Habit | |

**COMMENTS:**

Z

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# APPENDIX
# OBJECTIVE DESCRIPTION OF VARIETY
# PEA (*Pisum sativum*)

**Variety Name or Temporary Designation:** CEBECO 1145 (TOLEDO)

## LEAFLET CHARACTERISTICS:

[2] **Leaflet Type:** 1=Leafless 2=Semi 3=Normal

## STIPULE CHARACTERISTICS:

[3] **Color:**
1=Light-Green 2=Medium-Green 3=Dark-Green 4=Blue-Green 5=Yellow-Green 6=Other _____

**Please provide example varieties of similar specified color or check varieties and stipule color.**

| Variety Name | Stipule Color |
|---|---|
| Solara | 3 |

| Variety Name | Stipule Color |
|---|---|
| Montana | 2 |

| Variety Name | Stipule Color |
|---|---|
| | |

[2] **Size:** 1=Small 2=Medium 3=Large

**Please provide example varieties of similar specified size or check varieties and stipule size.**

| Variety Name | Stipule Size |
|---|---|
| Montana | 2 |

| Variety Name | Stipule Size |
|---|---|
| Solara | 2 |

| Variety Name | Stipule Size |
|---|---|
| | |

**OTHER CHARACTERISTICS:** Describe other characteristics that may aid in identification.

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EXHIBIT D

**Additional Description of CEBECO 1145 (Toledo), field pea**

In our own trials **CEBECO 1145** is closest to Solara. Differences are observed using electroforesis.

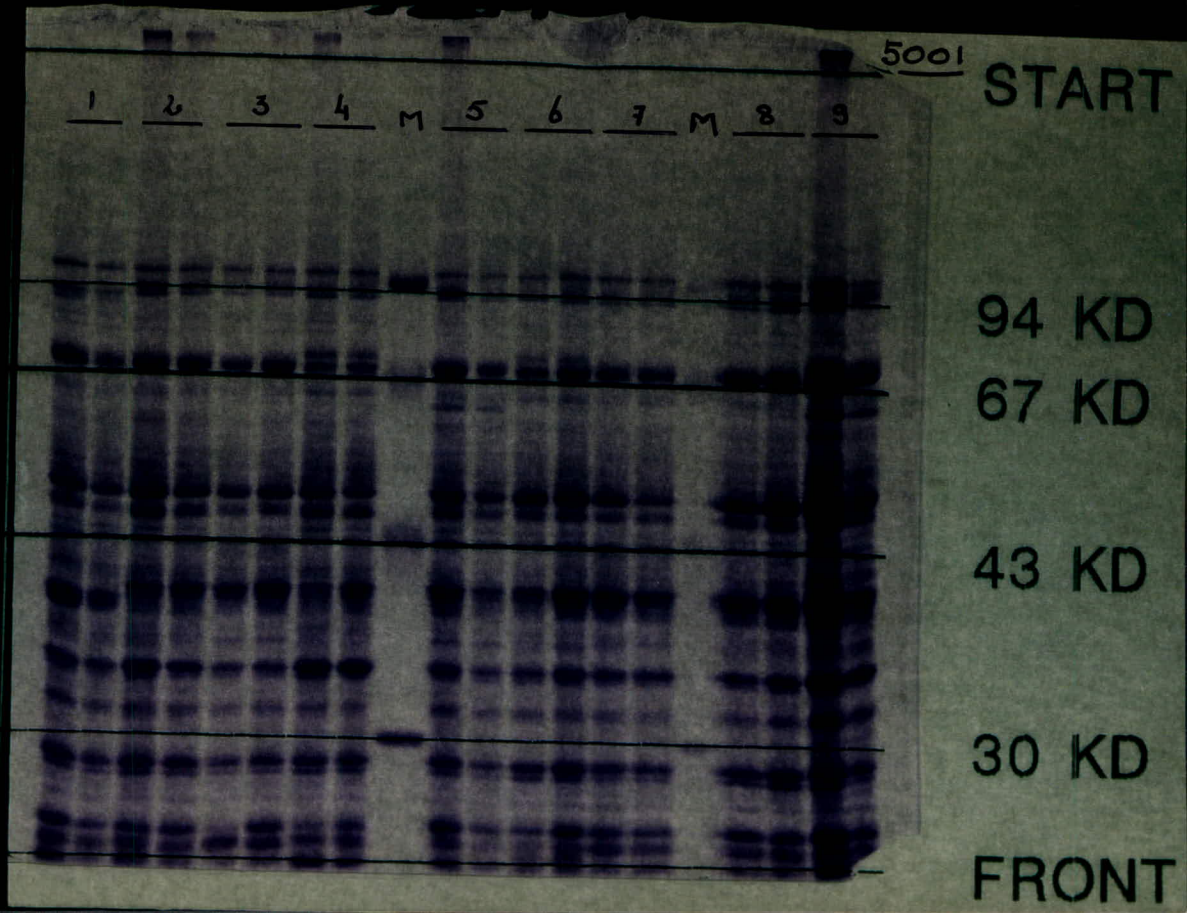
Attached you will find:
- a copy of the method used;
- a photograph of the gel.

Legenda:
---
1. Carrera
2. Cebeco 1141
3. Cebeco 1154
4. Cebeco 1145
M. Markers
5. Espace
6. Astina
7. Solara
M. Markers
8. Renata
9. Impala

---

9

ISO 9001


5001
START
1
2
3
4
M
5
6
7
M
8
9
94 KD
67 KD
43 KD
30 KD
FRONT

Please address comments to ASPP@sheridan.com

# HANDBOOK OF VARIETY TESTING



## ELECTROPHORESIS HANDBOOK: VARIETY IDENTIFICATION

Chief editor:

Dr. Robert J. Cooke, NIAB, Cambridge, UK

ISTA Variety Commitee

Chairman: Dr. R. C. Payne

Published by
The International Seed Testing Association
P.O. Box 412, CH-8046 Zurich, Switzerland, 1992

# Preface

The ISTA Variety Committee was asked to review the "Testing for Genuiness of Cultivar Handbook" that was published a number of years ago and determine if this handbook should be revised. The results of a questionnaire sent to ISTA member stations by Mr. van der Burg and a survey of ISTA Variety Committee members indicated that many new techniques and testing procedures had been developed since the "Testing for Genuiness of Cultivar Handbook" was published. Therefore, the Variety Committee decided that a revision of the existing handbook was appropriate. It was further decided that the revised handbook should have five chapters, with each chapter covering a different approach to variety testing. The chapters are entitled "Rapid Chemical Identification Techniques", "Growth Chamber – Greenhouse Testing Procedures", "Laboratory tests for Variety Determination with Fungal Pathogens", "Electrophoresis Testing" and "New Technologies for Variety Testing".

The development of new testing procedures and techniques is causing variety testing to become more and more specialised with individuals using only one or two testing procedures. It has become apparent that some people may be interested in the information in only one or two of the handbook chapters. Therefore, it was decided that each of the five chapters should be published as a separate handbook and be offered for sale either individually or as a set of five handbooks.

The electrophoretic methods presented in detail in this Handbook are of three types, namely:

Type (S1) – those that are already in the ISTA Rules;

Type (S2) – those that have been standardised by the ISTA Electrophoresis Working Group and proposed for incorporation into the Rules;

Type (S3) – those that are undergoing comparative testing by the ISTA Electrophoresis Working Group for the purpose of standardisation.

# Content

### 2.5.2 Peas and Ryegrass (*Pisum sativum* L. and *Lolium* spp.)

The Electrophoresis Working Group considered suitable methods for identifying varieties of peas and ryegrass from the seed. The most widely used and suitable methods were those using SDS-PAGE to analyse 'total' SDS-soluble seed proteins. The method described below is basically a 'Laemmli' system (7), adapted for use with seed proteins. It has been standardised by the Working Group and is proposed for inclusion in the Rules. It can therefore be designated a Type S2 procedure (see Preface).

## B. Proposed ISTA Standard Reference Method for the Identification of Varieties of Peas and Ryegrass by SDS-PAGE

### B. 1. Principle

Seed proteins are extracted from individual pea seeds or from ryegrass seed meals, treated with SDS and separated using a discontinuous SDS-PAGE procedure. The pattern of protein bands found on the gel is characteristic of a variety.

### B. 2. Chemicals

*All chemicals should be 'Analytical Reagent' grade or equivalent.*

Acrylamide ('specially purified for electrophoresis')
Bisacrylamide ('specially purified for electrophoresis') (BIS)
Tris (Tris (hydroxymethyl) methylamine)
Glycine
Hydrochloric acid (HCl)
Sodium dodecyl sulphate (SDS)
Glycerol
2-mercaptoethanol
Dimethylformamide (DMF)
Ammonium persulphate (APS) (*or* riboflavin)
TEMED (NNN'N'-tetramethylethylenediamine)
Methanol
Glacial acetic acid
Trichloroacetic acid (TCA)
PAGE Blue G-90 (*or* PAGE Blue 83). (*or* any reagent equivalent to the 'Coomassie Brilliant Blue' G or R series of dyes)
Bromophenol Blue

### B. 3. Solutions

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#### B. 3.1 Stock gel buffer (main or resolving gel) 1M Tris HCl, pH 8.8

| | |
|---|---|
| Tris | 121.1 g |

*is dissolved in about 750 ml of distilled water, adjusted to pH 8.8 by adding dropwise*

| | |
|---|---|
| 1M HCl (approx 90 ml concentrated HCl per litre distilled water) | 15 ml approx. |

*Make up to 1 litre. This can be stored at 4° C.*

#### B. 3.2 Stock gel buffer (stacking gel) 1M Tris HCl, pH 6.8

| | |
|---|---|
| Tris | 30.0 g |

*is dissolved in about 200 ml of distilled water, adjusted to pH 6.8 by adding dropwise*

| | |
|---|---|
| HCl (concentrated) | 8 ml (approx) |

*then*

1M HCl

*Make up to 250 ml. This can be stored at 4° C.*

#### B. 3.3 Stock SDS solution

| | |
|---|---|
| SDS | 10 g |

*is dissolved in distilled water, stirring under gentle heating. Make up to 100 ml.*
*This should be stored at room temperature – if the SDS comes out of solution, it can be re-dissolved by gentle heating.*

#### B. 3.4 1% ammonium persulphate solution

| | |
|---|---|
| Ammonium persulphate | 0.1g |

*is dissolved in 10 ml distilled water.*
*This must be prepared freshly on each occasion, immediately prior to use.*

### B.3.5 Stock sample extraction buffer solution

| | |
|---|---|
| Stacking gel buffer (B.3.2) | 12.5 ml |
| Glycerol | 20 ml |
| Distilled water | 24.1 ml |
| SDS | 4 g |
| Bromophenol blue (optional) | 12 mg |

*Mix and store at room temperature. If the SDS comes out of solution it can be re-dissolved by gentle heating.*

### B.3.6 Electrophoresis tank buffer (running buffer)

| | |
|---|---|
| Tris | 3.0 g |
| Glycine | 14.1 g |
| SDS | 1.0 g |
| Distilled water to make up to | 1 l |

*(It may be necessary to warm the solution gently to dissolve the SDS.)*
*A sufficient volume to fill the electrophoresis apparatus in use (top and bottom chamber) should be freshly prepared.*

### B. 3.7 Gel fixing solution

| | |
|---|---|
| Methanol | 400 ml |
| glacial acetic acid | 100 ml |

*Mix and make up to 1 litre. About 200 ml is needed per gel.*
*(Note: it is possible to use TCA at a final concentration of 15–20% (B.3.8) in place of glacial acetic acid.)*

### B. 3.8 Gel staining solution.

| | | |
|---|---|---|
| a) | TCA (15% trichloroacetic acid) | 375 g |
| | Water to make up to 2.5 l | |
| b) | 1% PAGE Blue or equivalent | 1 g |
| | Methanol. | 100 ml |

*200 ml of a) plus 10 ml of b) is sufficient for staining 1 gel.*

### B. 4. Protein extraction

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#### B. 4.1 Sample size

For peas, as a guideline it is recommended that 100 individual seeds are used. Very precise estimates of varietal purity may require a larger sample. If a comparison is being made with a standard value, sequential testing using batches of 50 seeds can be undertaken in order to minimise the workload. A simple check on the identity of a single major constituent of a seed lot can be done using less than 50 seeds.

For ryegrass, a bulked sample of seeds is analysed. In most cases, whilst this will serve to identify seed lots, it will not permit the detection of admixtures of two or more varieties.

#### B. 4.2 Peas

Finely ground pea cotyledon material is prepared from individual seeds, using an electric blender. A pestle and mortar (or similar device) can be used if preferred. Diluted sample extraction buffer is prepared by diluting the stock sample extraction buffer (B.3.5) in the following ratio – 17 buffer: 3 mercaptoethanol: 40 distilled water (only make up a volume of the diluted extractant sufficient to be used within a day). The finely ground seed meal is extracted with diluted sample extraction buffer in the ratio 40 mg/1.0 ml, using 1.5 ml polypropylene micro-centrifuge tubes. The samples are left for 1 hour at room temperature, resuspended using a vortex mixer and heated for 10 minutes in a boiling water bath. (A small slit can be made in the caps of the tubes to prevent a build-up of pressure.) After cooling, the tubes are centrifuged at 18000 x g for 5 minutes and the clarified supernatants used for electrophoresis.

#### B. 4.3 Ryegrass

Seed meals for analysis are prepared by passing 0.5 g – 2.0 g of seed through a hammer mill. If preferred, a rotor type electric coffee grinder or other blender can be used. Diluted extraction buffer is prepared by diluting the stock sample extraction buffer (B.3.5) in the following ratio – 17 buffer: 6 mercaptoethanol: 10 dimethylformamide: 17 distilled water (only make a volume of this extractant sufficient to be used within a day). The seed meal is extracted with diluted sample extraction buffer in the ratio of 80 mg/1.0 ml and subsequently treated exactly as above (B.4.2).



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## B. 5. Gel preparation

The clean and dry gel cassettes are assembled according to the type of equipment in use. (*Note*: if adhesive sealing tape is used in the system it is advisable to prepare the cassettes at least one day in advance to allow the tape to 'age' and adhere more tightly.) Many types of vertical electrophoresis equipment have been found to be suitable. It is strongly recommended that a gel thickness of 1.5 mm or less is used, as this seems to give better results. The following instructions are for the preparation of a 12.5% acrylamide main gel and a 5% stacking gel.

### B. 5.1 Main (resolving) gel

*For 4 slabgels. (180 x 140 x 1.5 mm)*

*De-gas in a Buchner flask*

| | | |
|---|---|---|
| **Gel solution** 86.25 ml | | |
| | Acrylamide | 19.6 g |
| | BIS | 0.26 g |
| | Distilled water | |
| | *to make up to 90 ml* | |
| 1M Tris pH 8.8 (B.3.1) | | 56.4 ml |

*Add*

| | |
|---|---|
| 1% APS (B.3.4) | 3.75 ml |
| 10% SDS (B.3.3) | 1.5 ml |
| TEMED (full strength) | 75 µl |

*Mix carefully, do not cause 'foaming'. Pour slowly.*

If appropriate to the type of equipment, a 25 ml disposable syringe and needle can be used to pour the gel mixture into the cassette. The gel should be poured to a height which leaves room for a 3–4 cm layer of stacking gel. The gel surface is carefully overlayed with a 1 cm layer of distilled water (or isopropanol), using a Pasteur pipette or syringe and the gel is then left to polymerise (about 1 hour).

*(Note: if de-gassing of the gel mixture is a problem, it is possible to eliminate this step and use a 3-times higher concentration of APS (ie 3.75 ml of a 3% solution [0.3 g dissolved in 10 ml of distilled water]).*

### B. 5.2 Stacking gel

*(For four slabs, as in B.5.1)*

*Remove the overlayed water (or isopropanol) from the surface of the main gel (with a Pasteur pipette). Rinse the surface briefly with*

Stacking gel stock buffer (B.3.2) diluted 1:8

*Drain carefully and dry using filter paper.*
*De-gas (in a Buchner Flask)*

| | | |
|---|---|---|
| **Gel solution** 67.2 ml | | |
| | Acrylamide | 4 g |
| | BIS | 0.07 g |
| | Distilled Water | |
| | *to make up to 67.2 ml* | |
| 1M Tris buffer pH 6.8 (B.3.2) | | 10 ml |

*Add*

| | |
|---|---|
| 1% APS (B.3.4) | 3.0 ml |
| 10% SDS (B.3.3) | 0.8 ml |
| TEMED (full strength) | 80 µl |

The stacking gel is poured (using a syringe as before, if appropriate) to the top of the gel cassette and an acrylic well-forming 'comb' is inserted, ensuring that no air-bubbles are trapped beneath the 'teeth'. The gel is allowed to polymerise (about 1 hour). Again, de-gassing can be omitted if a higher concentration of APS is used. It is recommended that 3.0 ml of a 2% solution (0.2 g in 10 ml of distilled water) should be sufficient.

As an alternative polymerisation system for the stacking gel, it is possible to use 0.008% riboflavin solution (freshly prepared), in place of APS. Polymerisation should occur if the gels are left in the light, but it may be necessary to use a UV lamp. The precise volume to use should be determined by experimentation, to give a polymerisation time of 30-60 minutes. However, as a guide, about 7.5 ml of riboflavin should be used per 50 ml of stacking gel mixture.

## B. 6. Electrophoresis

Remove the acrylic comb from the stacking gel with care, as this gel is rather soft. Wash the resultant wells. Partially fill them with a sufficient volume of tank buffer (B.3.6). The samples are loaded into the wells using a syringe. The gel thickness and the size of the wells largely determine the volume of extract which is loaded. As a guide between 5 and 15 µl is appropriate in most cases. If required, bromophenol blue (5 µl of a 1% aqueous solution containing 10% glycerol) can be added to a few wells, to act as a marker (this can also be incorporated into the sample extraction buffer, B. 3.5). If the gel cassette is sealed with adhesive tape, this is removed from the lower (bottom) side only. The wells are filled with tank buffer (as above), taking care not to disturb the samples. The gel

is placed in the tank and electrophoresis carried out at 25 mA per gel until the tracking dye has migrated through the stacking gel and then at 45mA per gel until the bromophenol blue is at the bottom of the gel. The temperature should be maintained at 15-20° C. if possible. by circulating tap-water (or coolant) through the tank buffer.

### B. 7. Fixing and staining

Several different approaches can be used for fixing and staining the proteins. If results are not required very rapidly, then at the end of the electrophoresis, the gel is removed from the tank, taken from the cassette and incubated in fixing solution (B. 3.6), with slow shaking, for at least 1 hour. The gel is rinsed in distilled water (5 mins) and then stained by incubation (at least 2 hours, usually overnight) in gel staining solution (B. 3.7). When properly stained, the gel is rinsed in distilled water for 2–3 hours (TCA can be added if the background is very blue) and then sealed in a polythene bag for examination or photography. Gels can be stored for many months at 4°C, if sealed properly. For more rapid staining, the gel can be fixed and stained at a higher temperature (80° C) in an oven for 30 mins and then, following cooling, destained in a solution containing 10% glacial acetic acid and 35% ethanol for a further 30–60 mins, with shaking.

Typical results produced using this procedure and methods for nomenclature of protein bands are presented in Section 3 of the Handbook. It should be noted that SDS-PAGE has been utilised for variety identification in a wide range of other species including cereals (see references 3.4.15 for further details).

## 2.6 Standard Procedures for Starch Gel Electrophoresis of Enzymes from Maize (*Zea mays* L.)

ISTA has not yet carried out any collaborative testing or assessment of methods suitable for maize variety identification. A method for testing hybrid purity in maize, using IEF to analyse zeins, is undergoing a comparative test, with promising initial results (2.7). However, there already exist methods which are so well established and widely used for maize that they can probably be considered as standards. These methods, which utilise starch gel electrophoresis to separate various isozymes from maize coleoptiles, have been well described in an excellent and comprehensive handbook 'Techniques and Scoring Procedures for Starch Gel Electrophoresis of Enzymes from Maize (*Zea mays L.*)'. This publication is available from Professor C W Stuber as Technical Bulletin number 286 from the North Carolina Agricultural Research Service, North Carolina State University, Raleigh, North Carolina 27695, USA. The methods are also to be included in the AOSA Cultivar Purity Testing Handbook (to be published in 1991).

The starch gel electrophoresis techniques examine a range of enzymes extracted from maize coleoptile tissue. There is information available concerning 21 enzymes (40 loci), separated using one of six recommended gel and buffer systems. The chromosomal location and genetic control of most of the enzymes are well-documented, enabling the gel patterns to be interpreted in genetic terms. For more information, it is recommended that readers contact Prof. Stuber, or a member of the Electrophoresis Working Group such as Mireille Bourgoin or Prof. Miller B. McDonald.

## 2.7 Method for the Measurement of Hybrid Purity and for Variety Identification of Maize by Ultrathin-layer IEF

This procedure is currently undergoing examination by the Electrophoresis Working Group and hence is designated as a Type S3 method (see Preface) (8).

### 2.7.1 Principle

The alcohol-soluble proteins (zeins) are extracted from individual seeds and separated by IEF in ultrathin-layer gels. The pattern of protein bands found on the gel is characteristic of a variety or an inbred line. Also, there may be found one or more bands in the male parent, lacking in the female parent and present in the hybrid. These bands could be used as marker bands for the identification of hybrids.

Ultrathin gels are very economical. Gels can be run at higher voltages with shorter running times and faster staining.

### 2.7.2 Apparatus and Equipment

The 'Desaphor' electrophoresis apparatus (Desaga) and the Pharmacia-LKB 'Multidrive XL' (3500 V) power supply have been successfully used, but any suitable horizontal electrophoresis system should give comparable results.

### 2.7.3 Chemicals

*All chemicals should be "Analytical Reagent" grade or equivalent.*

2-Chloroethanol
Acrylamide (purified)
Bisacrylamide (purified) (BIS)

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U.S. DEPARTMENT OF AGRICULTURE
AGRICULTURAL MARKETING SERVICE
SCIENCE AND TECHNOLOGY DIVISION - PLANT VARIETY PROTECTION OFFICE

# EXHIBIT E
# STATEMENT OF THE BASIS OF OWNERSHIP

*The following statements are made in accordance with the Privacy Act of 1974 (5 U.S.C. 552a) and the Paperwork Reduction Act (PRA) of 1995.*

*Application is required in order to determine if a plant variety protection certificate is to be issued (7 U.S.C. 2421). Information is held confidential until certificate is issued (7 U.S.C. 2426).*

1. NAME OF APPLICANT(S)
CEBECO ZADEN B.V.
P.O.Box 10000,
5250 GA VLIJMEN - HOLLAND

2. TEMPORARY DESIGNATION OR EXPERIMENTAL NUMBER
CEBECO 1145

3. VARIETY NAME
TOLEDO

4. ADDRESS *(Street and No., or R.F.D. No., City, State, and ZIP Code, and Country)*
I.S.I.
POB 168
Halsey, Oregon 97348- USA

5. TELEPHONE *(include area code)*
+31 73 51 88555

6. FAX *(include area code)*
+31 73 51 88 666

7. PVPO NUMBER
9900018

8. Does the applicant own all rights to the variety? *Mark an "X" in appropriate block.* If no, please explain. [X] YES [ ] NO

9. Is the applicant (individual or company) a U.S. national or U.S. based company? [ ] YES [X] NO
If no, give name of country The Netherlands

10. Is the applicant the original breeder? If no, please answer the following: [X] YES [ ] NO

a. If original rights to variety were owned by individual(s):
Is (are) the original breeder(s) a U.S. national(s)? If no, give name of country _____ [ ] YES [X] NO

b. If original rights to variety were owned by a company:
Is the original breeder(s) U.S. based company? If no, give name of country The Netherlands

11. Additional explantion on ownership *(If needed, use reverse for extra space):*

## PLEASE NOTE:

Plant variety protection can be afforded only to owners (not licensees) who meet one of the following criteria:

. If the rights to the variety are owned by the original breeder, that person must be a U.S. national, national of a UPOV member country, or national of a country which affords similar protection to nationals of the U.S. for the same genus and species.

. If the rights to the variety are owned by the company which employed the original breeder(s), the company must be U.S. based, owned by nationals of a UPOV member country, or owned by nationals of a country which affords similar protection to nationals of the U.S. for the same genus and species.

. If the *applicant is an owner* who is not the original breeder, both the original breeder and the applicant must meet one of the above criteria.

he original breeder may be the individual or company who directed final breeding. See Section 41(a)(2) of the Plant Variety Protection Act for finition.

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